THE STUDY OF PREVALENCE OF DENGUE SERO TYPE IN MADURAI BY MOLECULAR METHODS

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CERTIFICATE

This is to certify that the dissertation entitled **" THE STUDY OF PREVALENCE OF DENGUE SERO TYPE IN MADURAI BY MOLECULAR METHODS "** by **Dr. S. LALLITHA**, for M.D. Microbiology Examination, March 2007 under The Tamil Nadu Dr. M.G.R. Medical University is a bonafide work carried out under my direct supervision and guidance.

Director

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DECLARATION

I, **Dr. S. LALLITHA** declare that the dissertation titled **"THE STUDY OF PREVALENCE OF DENGUE SERO TYPE IN MADURAI BY MOLECULAR METHODS"** has been prepared by me.

This is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfilment of the requirement for the award of M.D. Degree, Branch IV (MICRO BIOLOGY) degree Examination to be held in MARCH 2007.

Place : Madurai

Date : **Dr. S. LALLITHA**

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CONTENTS

INTRODUCTION

The Arthropod-borne or arboviruses are a group of infectious agent that are transmitted by blood sucking arthropods from one vetebrate host to another. Replication in the vertebrate host produces sufficient viremia that will infect other blood feeding arthropods. The vector acquires a lifelong infection through ingestion of blood from a viremic vertebrate. The virus multiplies in the tissues of the arthropod without the evidence of disease or damage. Some arboviruses are maintained in nature by transovarian transmission in arthopods [75].

More than 500 viruses are now included in the international catalogue of arboviruses. Any haemophagus arthropod can serve as a vector of an arbrovirus. Recognised vectors include mosquitoes, ticks, sand flies, swallow bugs and possibly mites. Except for urban yellow fever, dengue and chikungunya virus, humans are typically dead end hosts for the viruses. Generally lower vertebrates such as avians and rodents serve as vertebrate hosts for a few of these viruses. The major Arboviral disease world wide are yellow fever, dengue, Japanese Encephalitis, St. Louis Encephalitis, Western Equine Encephalitis, Eastern Equine Encephalitis, Russian spring summer encephalitis, West nile and Sand fly fever. [29]

Overview of Arbo viral infection:

Diseases produced by arboviruses may be divided into three clinical syndromes.

1. Fever of an undifferentiated type with or without a maculopapular rash and usually benign.
2. Encephalitis often with high case fatality rate.
3. Hamorrhagic fevers : frequently severe and fatal

Dengue Infection:

Global prevalence of dengue, an arbo viral mosquito borne disease, has grown dramatically in recent decades giving rise to an escalating public health problem. In spite of extensive research done, many aspects of this disease remains obscure. Dealing with the uncontrolled expansion of dengue infection continues to pose a challenge. The first clinical report of dengue is attributed to Benjamin Rusk who coined the term ' break bone fever ' during the philedelpia epidemic in 1780. Dengue epidemics were reported through out the 19th and 20th centuries in Americas, Southern Europe, North Africa, The Eastern Mediterranean, Asia and Australia and various islands in the Indian Ocean, The south and central pacific and the Caribbean. Dengue fever (DF) or Dengue haemorrhagic fever (DHF) and have steadily increased in both incidence and distribution over the past 40 years. Annually it is estimated that there are 20 million cases of dengue infections resulting in around 24,000 deaths [64].

During the 1960s and 1970s, DHF/DSS progressively increased as a health problem, spreading from its primary location in major cities to smaller cities and towns in endemic countries. In many countries, Dengue fever and Dengue haemorrhagic fever are primarily a disease of children, since they represent the largest segment of susceptible individuals with the population at risk [76].

In India, dengue fever has been documented from the early part of last century. The virus was first isolated in Kolkatta in 1945. All four dengue sero types are present in India. In 1988, some cases of DHF were seen for the first time in children in Delhi. The first culture confirmed extensive epidemic of DHF, affected Delhi and adjoining areas during 1966. This was caused by DEN – 2 and affected mostly 5 to 20 years age group. India was affected by a much more wide spread dengue epidemic in 2003. During this epidemic, the most affected age group was 21 – 30 years. DHF is now a significant public health problem in most of the countries in the tropical areas of the South – East Asia and Western Pacific Regions. The disease is among the ten leading cause of hospitalization and death in children in at least eight tropical Asian countries [14, 30].

The Dengue Viruses

Dengue virus belongs to the family Flaviviridae. The four serotypes of dengue virus (designed DEN-1, DEN-2, DEN-3, DEN-4

etc.,) can be distinguished by serological methods. Infection in humans by one serotype produces life-long immunity against reinfection by that same serotype, but only temporary and partial protection against the other serotypes. They posses a single stranded, non segmented positive sense RNA approximately 11Kb long surrounded by an icosahedral nucleocapsid and covered by a lipid envelope. The size range from 37 – 50 nm in diameter. The genome is unfragmented. The RNA has 5' cap but lacks a Poly A 3' tail. The mature virion consists of 3 structural proteins – core (c), membrane associated (M) and envelope (E) and 7 non structural proteins. NSI (associates with infected cell's membrane), NS2a, NS2b, NS3 (perinuclear localization), NS4a, NS4b, and NS5 (RNA dependent RNA polymerase). The envelope glycoprotein is involved in the main biological function of the virus, it binds to the receptors on the host cells, allowing the virus to be transported through it. It is also associated with Haemagglutination, induction of neutralizing antibodies and protective immune response. The order of proteins encoded is 5'-C-prM(M)-E-NSI-NS2A-NS2B-NS3-NS4A-NS4B-NS4B-NS5-3'. Different strains of dengue virus may differ in their virulence in their pathogenic and epidemic potential [49, 76].

Transmission of Dengue viruses

Dengue viruses are transmitted to humans through the bite of infected mosquitoes principally by the bite of Aedes aegypti. Once

infected, the mosquitoes remain infected life long, transmitting the virus to susceptible individuals. Transovarian transmission can also occur. The viruses are known to cause infection only in humans [22].

The vector

Aedes aegypti (Ae. aegypti) is a tropical and subtropical species of mosquito found around the globe, usually between latitudes 35°N and 35°S, approximately corresponding to a winter isotherm of 10°C. Although Aedes aegypti has been found as far north as 45°N, such invasions have occurred during the warm season, and the mosquitoes have not survived the winters. Distribution of Ae. aegypti is also limited by altitude. It is usually not found above 1000m but has been reported at 2121m in India. Ae. aegypti is one of the most efficient mosquito vectors for arboviruses, because it is highly anthropophilic and thrives in close proximity to humans and often lives indoors. The female mosquitoes bite humans during the day. The virus multiplies in the salivary gland and when the mosquito bites humans for food the virus is injected into the host. Once infected, the mosquito hosts remain infective for life. Dengue outbreaks have also been attributed to Ae. albopictus, Ae. polynesiensis and several species of the Ae. scutellaris complex. Each of these species has its own particular geographical distribution; however, they are less efficient epidemic vectors than Ae. aegypti. While vertical (possibly transovarian) transmission of dengue viruses has been demonstrated in

both the laboratory and the field, the significance of this to maintenance of the virus has not been established. A factor complicating eradication of the vector is that Ae. aegypti egg can withstand long periods of desiccation, sometimes for more than a year [76].

The host

In humans each of the four dengue virus serotypes has been associated with DF and with DHF/DSS. DSS occurs frequently in two immunologically defined groups, children who have experienced a previous dengue infection and infants with wanning level of maternal dengue antibodies. The acute phase of infection follows an incubation of 3-14 days, lasts for 5-7 days and is followed by an immune response [9, 76].

Pathogenesis

Most primary infections are clinically silent. Dengue fever though debilitating is mostly self limiting. DHF and DSS occur more frequently due to sequential infection in person sensitized by prior exposure to a different serotype of dengue virus. The risk factors for DHF include the strain and serotype of the infectious virus, as well as the age, immune status and genetic predisposition of the patient. Case fatality and hospitalization rates due to DHF/DSS are highest in infants and elderly. DHF is distinguished from DF by the presence of increased vascular permeability. The targets for dengue virus replication are the cells of monocytes / macrophage lineage that bear Fc receptors(FcRs). In those

who had previous infection with heterologus dengue virus serotype, the secondary infection results in the production of non-protective (non-neutralizing) IgG antibodies that bind to the virion surface and through interaction with the Fc receptors, focus secondary dengue viruses on the target cell the result being enhanced infection. Due to similar antibody dependant enhancement (ADE), children in their first year of life who had acquired maternal Antibody with ADE characteristics, may develop DHF with primary infection. Activation of complement with profound depression of C_3 & C_5 is a constant finding in DHF/DSS. T cells response, especially that of cross reactive T cells play a role in the pathogenesis of DHF. The rapid release of cytokines and chemical mediators caused by T cell activation and lysis of infected monocytes, directly affects vascular endothelial cells resulting in plasma leakage and hemorrhage. Interferon Gamma up regulates expression of FC receptors and thereby increase antibody dependent enhancement of dengue virus replication.

Platelet defects may be both qualitative and quantitative, i.e. some circulating platelets during acute phase of DHF may be exhausted (incapable of normal function). Therefore even a patient with a platelet count > 1,00,000 still have a prolonged bleeding time [69, 12].

Internal organs

Liver: Focal necrosis of hepatic cells, swelling, appearance of

councilman bodies and hyaline necrosis of kupffer cells.

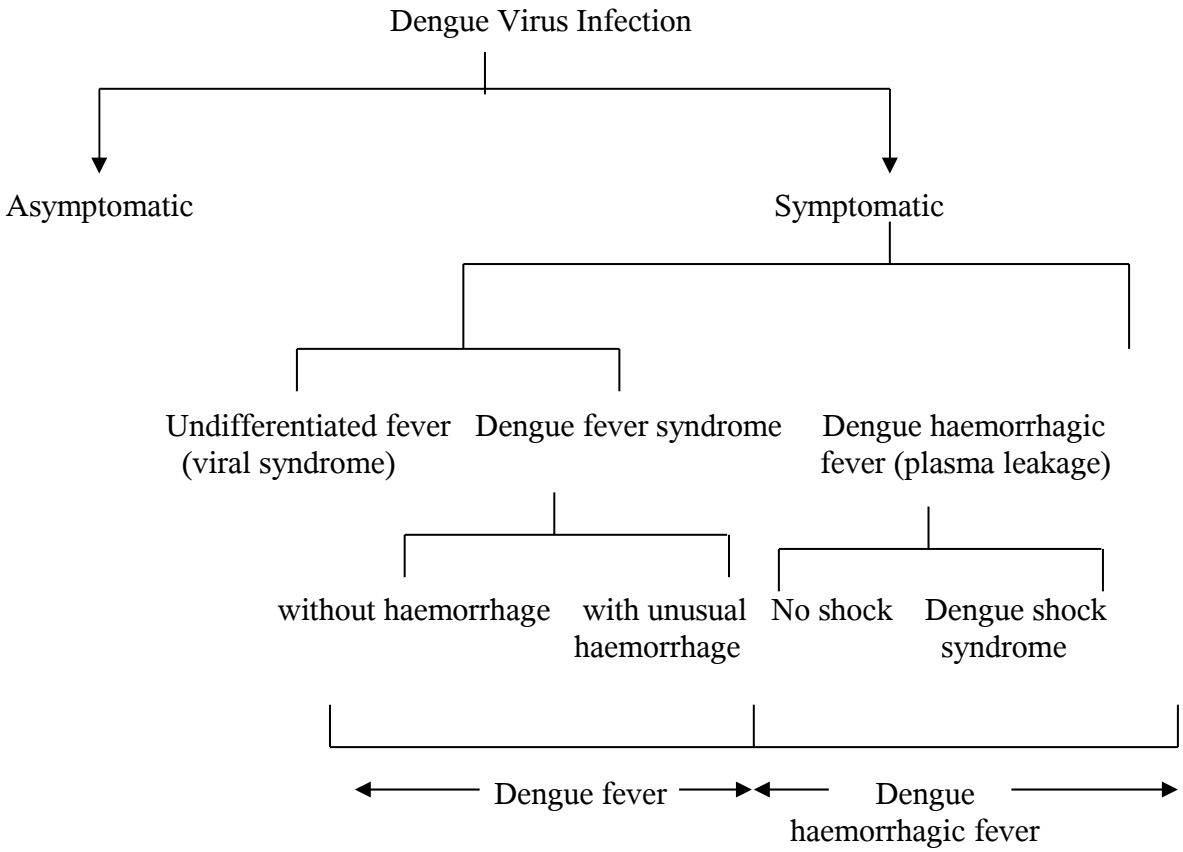
Bone Marrow: Depression of all hematopoietic cells was observed

which would rapidly improve as fever subsided

Kidney: Mild immune complex type of Glomerulo nephritis

Skin: Perivascular Oedema of the terminal micro vasculature of dermal papillae and infiltration of lymphocytes and monocytes. Deposition of serum complement, IgG, fibrinogen in vessel wall has also been described [76].

Manifestations of Dengue virus infection



Dengue Fever:

Clinical pictures depends on the age of the patient. Infants and young children may have indifferent febrile illness with rash. Older children and adults have mild febrile illness or fever of abrupt onset with 2 peaks., saddle back type of fever, severe headache, pain behind eyes, muscle and bone or joint pains, nausea and vomiting and rash. Leukopenia and thrombocytopenia may be observed

Dengue Haemorrhagic Fever

Characterised by four major clinical manifestation. High fever, haemorrhagic phenomenon and often hepatomegaly and circulatory

failure. Major patho physiological changes that determine the severity of the disease in DHF and DF is leakage of plasma, manifested as elevated haematocrit, a serous effusion or hypoproteinemia. Clinical presentation is characterized by sudden rise in temperature accompanied by facial flush, anorexia, vomiting, headache, muscle or bone and joint pain.

Signs: positive tourniquet test, mild hepatomegaly [28]

Dengue Shock Syndrome

The condition of patients who progress to shock suddenly deteriorates after fever of 2-7 days' duration. DSS is characterized by weak pulse with narrowing of pulse pressure and becomes fatal if not managed appropriately. Pleural effusion and ascites may be detected by physical examination and radiography. Convalescence in patient with corrected Dengue Shock Syndrome is short and uneventful. Course of DHF is approximately 7-10 days [76].

Serological diagnosis depends on the presence of IgM antibody or a rise in IgG antibody titre in paired acute and convalescent phase sera. IgM antibody becomes detectable during the acute phase of illness and 90% of patients are IgM positive by the sixth day after onset of symptoms. This antibody may be detectable for a median of about 60 days. Currently the most widely used IgM assay is capture ELISA (Enzyme Linked Immuno Sorbant Assay) [7].

Diagnostic approach

Detection of dengue virus by culture is the definitive diagnostic test, but practical consideration limits its use. Rate of virus isolation significantly improves in those samples collected within 6 days after the onset of disease.

Four isolation systems are routinely used [76]

1. **Intracerebral inoculation** into 1-3 days old suckling mice, mammalian cell cultures, inoculation of mosquito adult / larvae and use of mosquito cell culture.

2. **Detection of viral antigens**

Dengue viral antigen may be detected in acute sera and in peripheral blood mononuclear cells especially during febrile phase of illness. Antigen capture ELISA (Enzyme Linked Immuno Sorbent Assay) and IFA technique have been recommended for use.

3. **Detection of Viral Nucleic Acid**

- PCR based techniques
- Hybridization probes detects viral nucleic acid with cloned hybridized probes.

RT-PCR amplification may provide a rapid serotype specific diagnosis. This method is rapid, simple and can be used for detection of viral RNA in human clinical samples, autopsy tissues or mosquitoes.

4. **Serology**

Less specific than isolation of viruses

a. ELISA – Dengue specific IgM or IgG ELISA
b. HAI – Heam agglutination Inhibition
c. NT – Neutralization Test
d. CFT – Complement Fixation Test

Management

Dengue fever:

Supportive - Bed rest during febrile period, antipyretic, electrolyte, fluid replacement if there is vomiting, sweating, thirst or diarrhea.

Degnue Haemorrhagic Fever / Dengue Shock Syndrome:

Immediate evaluation of vital signs and the degree of haemo concentration, dehydration and electrolyte imbalance.

Blood transfusion / transfusion of platelets – if there is bleeding.

Prevention of Dengue Infection

A. Vector Control

I. Antilarval Measure

a. Environmental Control
b. Chemical control
c. Biological control

II. Anti adult measures

1. Residual sprays
2. Space sprays
3. Genetic control

III. Protection against Mosquito bites

a. Mosquito Net

b. Repellents

B. Dengue Vaccines [69]

Approaches towards development of Dengue Vaccines

The following technologies were attempted to develop a vaccine against dengue

1. Live attenuated Tissue Culture Vaccine
2. Infectious clone technology
3. Synthesis of subunit vaccines
4. Alternative biotechnological based dengue vaccine candidates

Economic aspect of dengue

Children most frequently suffer from DHF/DSS with average hospital stay involves 5-10 days for severe cases. Intensive care is needed for severely ill patients. There is both direct and indirect costs for each dengue patients ranging from inconvenience to substantial cost for hospitalization and disruption of earning potential [76].

AIM AND OBJECTIVE OF THE STUDY

1. **To study the epidemiology of Dengue in Madurai by screening the suspected Dengue cases admitted at Government Rajaji Hospital, Madurai.**
2. **To study the sero prevalence of Dengue infection in these cases by subjecting the serum samples to IgM / IgG spot ELISA and IgM capture ELISA.**
3. **To isolate the Dengue virus from the IgM positive samples by inoculating into the suckling mice.**
4. **To confirm the presence of Dengue antigen in the brain of suckling mice by haemagglutination (HA) technique.**
5. **To confirm the presence of Dengue virus in the brain isolates by subjecting to RT-PCR.**
6. **To find the type of the Dengue virus by single tube multiplex PCR by sero type specific amplicons.**

REVIEW OF LITERATURE

Dengue fever and especially the more severe manifestation, Dengue haemorrhagic fever, ranks highly among new and newly emerging infectious disease in public health significance and is considered to be the most important of all the arthropod borne viral diseases (**WHO – 1997**) [76].

Most arboviruses are maintained in nature in cycles involving preferred hematophagous arthropod vectors and vertebrate hosts. The amplifying vertebrate host develops viremia of sufficient titer and duration to infect additional feeding vector species **(Watts et al 1973)** [75].

We owe to Benjamin Rush the first to describe the accurate clinical description of the dengue fever as is occurred during Philadelphia epidemic of 1780 **(Siler et al 1926 and Carey 1971)** [66,10].

Schlesinger 1977 [64] in his article mentioned that during 19th and 20th centuries extensive outbreaks were reported from tropical and sub tropical areas on all continents and from many sub continents and islands in the south pacific and Caribbean.

Gubler 1998 [27] stated that Dengue was a major public health problem globally. Manifested clinically in two forms viz. Dengue Heamorrhagic Fever (DHF) and Dengue Shock Syndromes (DSS),

leading to serious public health problem exacting heavy morbidity and mortality every year.

WHO 1997 [76] states that, this mosquito borne disease was the dreadful infectious disease after malaria, with an estimated 100 million cases of DF, 50,000 cases of DHF and 2,50,500 deaths annually.

By 1997 most of the countries of SEARO had experienced large out breaks of the disease, currently DF/DHF is endemic in Bangaladesh, India, Indonesia, Maldives, Myanmar, Srilanka and Thailand. Approximately 1.3 billion people are living in the endemic areas. Dengue Fever / Dengue Haemorrhagic Fever is widely prevalent in India and all the 4 sero types are found in the country. It is reported from 15 States / Union Territories since 1996. During 2003, there were 12750 cases with 217 deaths from dengue in the country. [**Government of India 2004**,[24] **Annual report 2003-2004, Ministry of Health and Family Welfare, New Delhi].**

In Southern India, Dengue was mainly an urban disease in the 1960's and 1970's associated with the container breeding vectors of Aedes aegypti. Many isolations of all the four sero types of dengue virus were made from pools of Aedes aegypti. During that time, there was little storage of water in villages and therefore Aedes species were scarce and dengue was absent in rural areas. Subsequently with the introduction of piped water supply, dengue made incursions to the rural areas of South

India and outbreaks have been reported. **[Carey et al., 1964 and Abdul Kader et al, 1997 [9, 1]]**

Rosen L. 1999 [60] in his article mentioned that demographic and societal changes such as population growth, urbanization and modern transportation contributed greatly to the increased incidence and geographic spread of dengue activity.

Gubler D.J. 2002 [29] in his study stated that the current epidemiologic situation in Latin America resembled that in South East Asia some years ago with co circulation of multiple serotypes in many countries and increased number of DF and DHF cases. During 2002, Latin American countries reported 71 million cases of DF with 7,17,000 cases of DHF including 225 deaths.

Guzman and Kouri 2002 [33] in their article stated that, Dengue Virus was a mosquito borne flavivirus with 4 sero types DEN1, DEN2, DEN3 and DEN4. Dengue had been found in more than 100 countries and 2.5 billion people lived in areas where dengue was endemic. Fifty to one hundred million cases of dengue infection were estimated to occur annually. Dengue caused by four sero types of Dengue virus was the most prevalent arthropod borne illness in humans ranging from the DF to DHF/DSS.

WHO, Dengue Haemorrhagic Fever 1997 [76] stated that the clinical features and severity of dengue infection was frequently

influenced by the age and genetic make up of the host, the sero type of the infecting virus and the prior history of dengue infection of the host.

Dengue virus is a positive stranded encapsulated RNA virus. The Genomic RNA is approximately 11Kb in length and is composed of three structural protein genes that encode and nucleocapsid or core protein (C), a membrane associated protein (M) and an Envelope protein (E) and seven non structural proteins. **[Malaviga G N et al 2004]** [49]

Burke et al 1988, Halstead S B et al 2002 [7, 35] in their article stated that the proteins were synthesized as a polyprotein of about 3000 amino acids that was proceeded contranslationally and posttranslationally by viral and host proteases. There were four distinct sero types, sero types 1 – 4. Infection induced a life long protective immunity to the homologus sero types but conferred only partial and transient protections against subsequent infection by other sero types. Instead, it had generally been accepted that secondary infection or infection with secondary or multiple infections with various dengue virus sero type was a major risk factor for DHF/DSS due to antibody dependent enhancement.

Thakara J.P. et al 2004 [72] in their study found concurrent infection with more than one sero type.

Srivastra et al [68] in his study mentioned that, the distribution of DHF and DSS in Asia had been particularly puzzling. Before 1989, DHF was common in South East Asia but rare in the Indian subcontinent,

despite the circulation of all four serotypes in both regions. After 1989, this pattern of disease had changed and regular epidemics of DHF were reported from several countries in the Indian sub-continent.

WHO 1999 [77], referred Dengue that was an endemic viral disease affecting tropical and sub tropical regions around the world predominantly in urban and semi urban areas. Dengue fever and its severe forms, Dengue haemorrhagic fever and Dengue Shock Syndrome were becoming important public health problems and were formally included within the disease port folio of the UNDP / World Bank / WHO special programme for Research and Training in tropical diseases by the joint co-ordination Board in June 1999.

Susan Shepherd et al [71] states that transmission of Dengue is by the bite of infected female Aedes mosquitoes (Extrinsic Incubation period 8-10 days). The main vectors are Aedes aegypti (a container breeding day biting mosquito) and Aedes albopictus. Humans and mosquitoes are the principal host of dengue virus. The mosquitoes remain infected for life. The viruses are known to cause illness only in humans. In the rain forests of Africa and Asia the virus is probably sustained through vertical (transovarial) transmission in the mosquito with periodic amplification in non- human primates.

Joshi et al 2002 [42] in his article described that experimentally transovarial transmission had been shown in Aedes aegypti. Mosquitoes

and these vector mosquitoes also played an important role in the maintenance of the virus in nature.

Gupta E et al 2005 [30] in his article mentioned that the epidemic of 2005 in Delhi involved the age group of 21-30 yrs. The author concluded that the intensive mosquito elimination programme after the 1996 epidemics, might have shifted the mosquito population towards non residential areas and there by infected mobile working population of the higher age group.

Mahadev P V M et al, 2004 NIV Pune [48] also had found Aedes aegypti as the vector in Maharashtra.

Sumodan et al 2003 [70]**, and Hiriyan J. Tewari et al** [40] in their Vector surveys carried out at Ernakulam and Kozhikode airport, identified Aedes albopictus as the vector.

Dengue fever is characterized by fever for 3-5 days duration, headache, muscle and joint pain and a rash, which is self-limited and from which patients usually recover completely. There is no specific treatment for DF and most forms of therapy are supportive in nature. DHF/DSS is characterized by the same signs and symptoms as classic DF, but it is followed by increased vascular permeability and haemmorhage which may lead to vascular collapse and death **(Burke et al 1988** [7]**)**

Rita Maria Riberio Nogueira et al 2002 [59] have conducted a study involving 1559 cases with febrile illness. The symptoms noted were headache, retrobulbar pain, myalgia, arthrlgia, rash and hemorrhagic manifestation.

The Text Book of **Harrisons, Principles of Internal Medicine** [39]**,** state that Dengue virus infection may be asymptomatic or may give rise to broad spectrum of effects such as undifferentiated fever, DHF and DSS. Patient with Dengue haemorrhagic fever have high fever, haemorrhagic manifestation, hepatomegaly and circulatory failure. Thrombocytopenia and haemoconcentration are constant features. Rare presentations includes cardiomyopathy, fulminant hepatic failure and unusual neurological presentation.

Once inoculated into the human host, dengue has an incubation period of 3-14 days. Following incubation for about 5-7, days acute febrile illness ensues. Recovery is usually complete by 7-10 days. DHF or DSS usually develops around the third to seventh day of illness, approximately at the time of defervesence. The major pathophysiological abnormalities that occur in DHF and DSS are plasma leakage and bleeding. Plasma leakage is caused by increased capillary permeability and may be manifested by haemoconcentration, as well as pleural effusion and ascites. Bleeding is caused by capillary fragility and thrombocytopenia and may present in various ways, ranging from

petechial skin hemorrhage to life threatening gastrointestinal bleeding. Most patients who develop DHF or DSS have had prior infection with one or more dengue serotype.

Philip Samuel et al [55] in an epidemic investigation reported that 100% cases presented with fever (83.3%), myalgia (70%), arthralgia (56.7%), headache (6.7%) and retro orbital pain (6.7%).

Gadia R R et al 2002 [21] in his study found that 87% patients had headache, 83% body ache, 74% vomiting, 47% arthralgia, 40% bleeding, 38% Rhinitis, 38% abdominal pain, 25% anorexia, 20% diarrhoea, 16% cough, 8% Vertigo, 4% Seizures and 3% coma.

Ramamurty N. et al 2004 [56] stated that among 78 cases presented as dengue fever, 21 cases presented with neurological symptoms of altered consciousness, Convulsions and seizures.

Manoj Kumar et al 2005 [51] found in his studies that the serotypes responsible for the Delhi outbreak were serotype 2 & 3. Most of the cases were presented with DF and sporadic cases with DHF

Malaviga G.N et al 2004 [49] in his study stated that annually an estimated 100 million cases of DF and half a million cases of DHF occured in the world with a case fatality of 0.5-3.5 % in Asian countries. 90% of DHF subjects were children under 15 yrs of age

Dar L, et al [14] in his studies stated that epidemics of DHF in Delhi, in 1996 affected mainly 5-20 yrs age group.

At present, the three basic methods used by most laboratories for the diagnosis of dengue virus infection are viral isolation and characterization, detection of the genomic sequence by Nucleic Acid amplification technology assay and detection of dengue virus specific antibodies. After the onset of illness, virus is found in serum or plasma, circulating blood cell and selected tissues for approximately 2-7 days **(WHO – 1997 [76])**.

Molecular diagnosis based on RT-PCR such as one step or nested PCR or Nucleic Acid Sequence Based Amplification (NASBA) has greatly replaced virus isolation, as the new standard for the detection of dengue virus in acute phase serum sample. For acute and convalescent phase sera, serological detection of antibodies based on capture 1gM & 1gG ELISA has become the new standard for the detection of primary and secondary dengue virus infection. **[Gubler.D.J. 1997 [26], Innis B.L. et al 1989 [41]]**

Lanciotti R.S. et al 1992 [47] and Rosario D et al 1998 [61] in his article described that Polymerase Chain Reaction (PCR) had been applied to dengue diagnosis with sera, tissue from fatal cases, mosquito pool, infected cell cultures and mosquito larvae. Several PCR protocols for dengue detection had been described that vary in the RNA extraction methods, genomic location of primers, specificity, sensitivity and methods to detect PCR products and to determine the sero types. Reverse

Transcriptase-Polymerase Chain Reactions had been provided to be one of the most important steps in the molecular diagnosis of dengue virus. A rapid assay had been developed followed by a second PCR with specific primers allowing sero type identification (DNA products) of different sizes according to the dengue sero type obtained. Virus isolation by cell culture and from mosquitoes remain the gold standard, but has been replaced by RT PCR method for rapid diagnosis.

Oliveria D.P.S et al 2003 [53] has showed that the molecular method based on RT-PCR had been combined with the cell culture methods to improve the sensitivity and reduce the time needed to identify the cultured virus studies.

The field of molecular diagnosis has changed significantly over the past decades leading to assays that are much more reliable for the detection and characterization of various pathogens. Several protocols are available among these. The two steps multiplex RT PCR protocols originally reported by **Lanciotti et al (1992)** [47] and later modified to a single step multiplex RT-PCR for the detection and typing of dengue virus by **Harris et al (1998)** [37] are well known. These assays used the dengue virus core to pre membrane region as the target sequence for dengue virus detection. They had the advantage of detecting and differentiating the four dengue virus serotypes analyzing the unique size of the amplicons in the agarose gel.

Domingo et al 2004 [18] in his study using RT nested PCR on 167 samples from febrile illness diagnosed classical dengue fever by WHO criteria. Sixteen of these cases were found positive by RT nested PCR, all the serum sample were collected during the first week after the onset of symptoms.

Manoj Kumar et al, NICD New Delhi 2003 [51] studied 32 serum samples from clinically suspected cases of DF/DHF. Tested by RT-PCR, 7 were positive, yielded amplification of 511bp C-PreM region of dengue virus. Further studies revealed that among the 7 isolates, 5 belonged to dengue-type 3 and 2 belonged to dengue type 2.

Soundravally R and Hoti S L, Pondicherry 2004 [67] Studied 129 samples from suspected dengue cases. RT-PCR was done, for forty positive cases. Fourteen samples yielded amplicon for the size of 290 bp, diagnostic of dengue sero type 3.

Guzman M.G et al (1996) [32] explained the uses of cultured mosquito cells such as Ap61, C6/36 cells, vero and BHK2 cells in Dengue diagnosis.

Dash PK Saxena, D R & D Gwalior 2003 [15] showed isolation of virus from RT PCR samples, in day old suckling mouse as well as C6/36 cells

Several methods have been described for the serological detection of dengue viruses including haemaggutination, neutralization test,

indirect immuno fluorescence, ELISA, Compliment Fixation Test, dot blotting, western blotting and rapid immuno chromatography test. Among these capture IgM / IgG ELISA and antigen coated indirect IgM/IgG ELISA are commonly used.

Branch S.L. et al 1999 [5] & Lam S.K. 1986 [46] used rapid kits with the principle of Immuno chromatography which were commercially available. Most of these kits simultaneously detected IgM & IgG antibodies to dengue virus in human whole blood, serum or plasma within 5-30 min.

N. Sathesh et al, CMC Vellore (2000) [62] had used rapid test by Pan - Bio Rapid Immuno Chromatographic card. Out of 154 serum samples tested, 35(22.71%) were positive. It was found that Immuno Chromatographic card test was 100% sensitive and was able to distinguish between primary and secondary infection.

Branch S.L et al 1999 [5], studied the serum samples from 62 patients with the same kit and found the sensitivity to be 83.98%.

Attempts to create conventional vaccines have been hampered by the lack of suitable experimental models, the need to provide protection against all four serotypes, simultaneously and the possible involvement of virus-specific immune response in DHF / DSS (**Stephensan J R 2006 [69]**).

The first candidate dengue vaccines were developed shortly after the virus was discovered (**Gubler D.J. 1998 [28]**)

Both the WHO based initiative for vaccines research (IVR) and paediatric dengue vaccine initiative (PDVI), are working to facilitate vaccine development. Chimeric vaccines have been generated, the most advanced being, chimeric vax dengue. In this vaccine, 17D yellow fever virion and envelope protein genes are replaced with those from the dengue viruses. Other chimeric vaccines with cell adapted DEN2 viruses or dengue viruses with engineered mutation as genetic back bone have also been developed. **(Halstead S B and Dean 2002 [35], Stephenson. J.R. 2005 [69] and Chaturvedi U.C. 2005 [11])**

Laboratory attenuated strains of each of the four dengue serotypes previously tested as monovalent vaccines in volunteers were combined and tested for immunogenicity, safety and reactogenicity in 16 dosage combination. Seven formulation met the serological criteria required for an expanded trail and three of these were sufficiently attenuated clinically to justify further testing **(Guzman M J, Kouri 1990)** [31].

The only way to prevent dengue virus acquisition is to avoid being bitten by a vector mosquito, wear protective clothing, preferably impregnated with permerthin insecticide and use of N1N1 diethyl-3-Methyl benzamide (DEET) containing mosquito repellents. The use of mosquito netting is of limited benefit as aedes are day biting mosquitoes. Elimination of the mosquito vector using indoor sprays, elimination of breeding grounds of the mosquitoes by not allowing them,

access to small accumulations of stagnant water around human habitats such as found in pots, old tubes, on any vessel capable of holding water, and to support community based vector control programme are other options available as control measures. (**Dengue Hemorrhage Fever., WHO, 1997** [76])

MATERIALS AND METHODS

This study was conducted at Government Rajaji Hospital, attached to Madurai Medical College, Madurai and Centre for Research in Medical Entomology, Madurai. The study period was for 8 months from November 2005 to June 2006 and the study population included 125 suspected cases of Dengue fever with 5 – 10 days duration. Ethical clearance was obtained from Hospital ethical committee chairman.

The inclusion criteria for the study population were

1. Acute febrile illness of 5-10 days duration with 2 or more of the following symptoms.
    a. Head ache
    b. Retro orbital pain
    c. Myalgia
    d. Arthralgia
    e. Rash
    f. Haemorrhagic Manifestation
    g. Leucopenia
2. Age group ranging from 6 months to 65 years

The exclusion criteria were

a. Febrile cases without the above symptoms
b. Children below 6 months and above 65 years (extremes of ages)
c. Haemorrhagic conditions without evidence of Dengue fever.

Collection and Transportation of samples:

The blood samples were collected from the patients admitted in Government Rajaji Hospital from both endemic and non endemic areas for dengue viruses. Aseptically, 2-5 ml of blood was collected in vials with screw caps. The caps were fixed with adhesive tape to prevent leakage during transport. The specimens were transported to the lab immediately in an ice box with proper labeling of the name of the patient, identification number and date of collection.

Processing of Samples

As soon as the samples were received in the laboratory, they were centrifuged and serum was separated. The serum samples were stored in the refrigerator at $4^{O}C$ and tested within a week, if there is a delay it should be stored at -20 ^{O}C.

The collected specimen were processed for

Methodology I

Detection of dengue IgM / IgG antibodies by BIAS-3 dengue IgG/IgM kit.

Methodology II

IgM capture ELISA

Methodology III

For the propagation and isolation of dengue virus by inoculating the IgM positive sera intra cerebrally in suckling mice and harvesting the brain material.

Methodology IV

Confirmation of the dengue antigen titre by Haemagglutination technique.

Methodology V

The harvested brain materials were subjected to RT - PCR for amplification of 511 bp C-PreM gene region of dengue virus.

Methodology VI

Single tube multiplex PCR analysis employing dengue sero type specific amplimers.

I. Detection of Dengue IgG / IgM antibodies by BIAS - 3

This is a two step assay for the differential detection of IgG and IgM antibodies to Dengue virus in serum. The test kit is a rapid membrane based screening test to differentially detect the presence of antibodies to dengue virus. This test is the newer generation lateral flow immunochromatographic type assay.

Principle

The test employs the use of two antibody binding proteins conjugated to colloidal gold particles and a unique combination of Dengue antigens immobilized on the membrane. Once the sample is added to the sample pad along with the diluent, the mixture passes bi-directionally through the antibody binding / gold complexes, which then binds the immunoglobulins in the sample. As these complex passes over the immobilized antigen on the membrane, if any antibody to Dengue (IgG/IgM) are present the antigen capture them in turn. This produces a pink / purple band in the Test zone of the device. The remaining complex continues to migrate to a control area on the test device and produces a pink / purple band in each Control area. This control bands indicate that the test has been performed properly.

Test Procedure:

The kits were removed from the pouches and kept in a clean surface. 5 µl of the serum sample was added to the centre hole indicated by an 'S' on the device. 6 drops of sample diluent was added to the central hole. Development of purple band was taken as positive result.

Dengue IgM capture ELISA

The qualitative immuno enzymatic determination of IgM class antibody against dengue virus is based on ELISA (Enzyme Linked Immuno Sorbent Assay)

Technique

Micro titre strip wells are pre coated with dengue virus antigen to bind the corresponding antibodies of the specimen. After washing the wells to remove all unbound sample material, add antigen – Mab tracer solution is added. This conjugate binds to the captured dengue virus specific antibodies.

The immune complex formed by the bound conjugate is visualized by adding tera methyl benzidine (TMB), substrate which gives a blue reaction product. The intensity of this product is proportional to the amount of dengue virus specific IgM antibodies in the specimen. Sulphuric acid is added to stop the reaction. This produces a yellow end point. Color absorbance at 450 nm is red using an ELISA micro well plate reader.

Procedure:

1. Sample dilution: As per kit instructions all samples were diluted 1:100 with IgM sample diluent. 10µl of sample was diluted with 1ml of IgM. Samples were diluted in tubes to obtain a 1:100 dilution and thoroughly mixed in the vortex mixer.
2. Dilute the antigen provided, with antigen diluent. 10µl of antigen is added to 2.5ml of antigen diluent.

3. Remove the required volume of diluted antigen and mix with an equal volume of Mab tracer in a clean glass or plastic vial. Mix gently and leave at room temperature (20-25^{O}C) until use.

Methodology:

One well was allotted for the substrate blank, two well for negative control and one well for positive control.

The specimens as well as the positive and negative controls were added in to their respective wells. One well was left for substrate blank.

The wells were covered with the foil supplied in the kit. This was incubated for 1 hour at 37^{O}C. After this step, the foil was removed, the contents aspirated from the well and washed 6 times with wash buffer. The remaining fluid in the well was removed by tapping on tissue paper.

100µl of dengue virus antigen Mab tracer solution was added into all the wells except for the blank well and covered with foil. This was incubated for 1 hour at 37^{O}C. At the end of 1 hour, the wells were washed 6 times with diluted wash buffer. 100µl of TMB substrate solution was added to all the wells and this was incubated for 10 minutes at room temperature in the dark and blue colour developed. 100µl of stop solution was added to all the wells, the blue color developed during the incubation turned into yellow. The absorbance of the specimen at 450 nm within 30 minutes after the addition of stop solution was read in

ELISA reader. The absorbance value of all the wells at 450 nm were recorded for each specimen.

Calculations:

The average absorbance of the triplicate of the cut of calibrator was calculated, which was the cut off value.

Interpretation of Results:

A positive results (≥ 11 PANBIO Units) is indicative of either primary or secondary infection.

III. Isolation of Dengue Virus:

Specimen – Serum

Animals used were Swiss Mice (1 – 2 days) Sucking Mice

Animal Inoculation:

Virus Isolation:

Attempts were made to isolate the virus by inoculating the serum sample in 0 to 2 days old suckling mice (swiss mice), using 1ml tuberculin syringe with 25gauge needle. About 0.01ml – 0.02ml of serum was inoculated slightly lateral to the midline and into the mid portion of the lateral hemisphere. Each sample was inoculated in 6 – 8 mice in a litter.

After inoculation, the mice were observed daily for the sickness. Mice that die in fewer than 24 hours were discarded. After 24 hours, the mice were observed twice a day for any signs of abnormal behaviour. Signs were absence of milk in stomach, dark color, wasting, unusual activity, tremors, seizures or prostration.

Mice that developed sickness were euthanized, and the brain was harvested by removing the skull cap aseptically with scissors. The harvested mice brains were stored in sterile borate saline and kept at minus 70^{O}C until use.

IV. Haemagglutination Test (for confirmation of Dengue Antigen Titre)

A wide variety of animal viruses has been shown to posses the capacity of adsorbing to red cells and in many cases causing agglutination of these red cells. This haemagglutination test furnished a relatively simple, quick, convenient and fairly quantitative way of detecting, identifying and titrating viruses.

Procedure:

Mark the micro titre plate with the glass marking pencil giving different dilution of antigens.

With a 0.05ml dropper add one drop of 0.4% BABS to each well.

Keep plate in refrigerator for chilling.

Keep seven 0.05ml micro diluters with tips immersed in 0.9%

NaCl in refrigerator for chilling.

Dispense 0.9 ml of 0.4% BABS in a tube in an ice bath.

Add 0.1ml of antigen and mix thoroughly (1:10 dil. of antigen)

Remove plate and microdiluter from the refrigerator.

Blot 0.05ml micro diluters on blotting paper, dip the tips in tube with 1:10 antigen and place in each of wells marked 1:20 in the plate.

With the microdiluters, prepare serial two fold dilutions of antigen through well 10 by swirling the microdiluters 10-15 times (1:10240).

Add 2.4 ml of Virus Adjusting Diluent (VAD) 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2 to labeled tubes.

Add 0.1 ml of 10% RBC to each tube and mix well (This gives a dilution of 0.4% RBC)

With a 0.05 ml dropper pipette add one drop of RBC to corresponding row.

Tap plate or use shaker to mix cells and antigen dilutions.

Cover the plate and incubate at room temperature until cells in the wells to cell controls settle and form a button (usually about ½ hour)

Read and record the titration.

Complete agglutination consists of a layer of uniformly agglutinated cells covering the entire lower surface of the well. A negative pattern consists of a compact, sharply demarcated button of sedimented cells in the centre of the well, the pattern being in identical to that seen in the control wells.

V. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Equipments Needed

- ❖ Programmable thermal cycler
- ❖ Electrophoresis unit
- ❖ Power pack
- ❖ Agarose gel electrophoresis tank, tray and combs
- ❖ UV transilluminator
- ❖ Dark room photographic equipment
- ❖ Gel doc
- ❖ UV face shields
- ❖ Set of micropipettes
- ❖ Sterile microfuge tubes (1.5ml and 0.5ml)
- ❖ Sterile tubes for thermal cycler
- ❖ Sterile micro pipette tips
- ❖ Ice bucket

Reagents Required

- Template RNA
- Oligonucleotide primer
    - R T primer, Forward primer, Reverse primer
- RT buffer
- Taq polymerase
- Magnesium chloride
- d NTP mix
- Agarose
- IXTAE buffer

IDENTIFICATION OF VIRUS BY RT-PCR

RNA Extraction for RT-PCR

The total RNA of virus infected mouse brain was extracted using TRIZOL reagent as per the manufacturer's instructions.

Homogenization:

750 µl of Trizol reagent was added to 250µl of brain suspension (50-100 mg tissue) and the homogenate was incubated for 10 minutes at room temperature. Two hundred microlitre of cholorform was added and mixed vigorously. The samples were incubated for 2-3 minutes at room temperature and centrifuged at 10,000 rpm (12,000g) for 15 minutes at $4^{O}C$ in a refrigerated centrifuge.

RNA Precipitation:

The colourless upperface was collected and transferred to another sterile microcentrifuge tube. 0.5ml of isopropanol was added and incubated for 5-10 minutes at room temperature. Samples were centrifuged at 10,000 rpm for 10 minutes at 4^{O}C.

RNA wash:

The supernatent was removed and the pellet was suspended in 1ml of 75% Ethanol and centrifuged at 5000 rpm for 5-10 minutes at 4^{O}C.

Solubilization:

The RNA pellet was air dried for 5-10 minutes and dissolved in 20-30 µl of sterile milliQwater. The samples were incubated at 60 ^{O}C to 65 ^{O}C for 10 minutes.

Virus Identification using RT-PCR:

Reverse transcriptase PCR (RT-PCT) has been developed for a number of RNA viruses in recent years and has the potential to revolutionize laboratory diagnosis. RT-PCR provides a rapid serotype-specific diagnosis. The method is rapid, sensitive, simple, and reproducible if properly controlled and could be used to detect viral RNA in human clinical samples, autopsy tissues, or mosquito samples.

Protocol:

RT – PCR was carried out as per Lanciotti et al 1992, virus capsid region was amplified by using specific primers.

The virus specific primers were synthesized commercially and the sequences were obtained from the published reports.

1. 43µl of Rnase free H_2O and 1µl of reverse primer (DCP-B gene specific) and 1µl of gene specific forward primer (DCP-A) was added to the beads i.e. commercially available RT PCR kit. The primers were developed commercially (Q1AGEN).

2. To the tubes, 5µl of RNA Template of each sample was added.

3. All the PCR tubes were kept in Thermocycler (Master cycler, eppendoff) and the RT-PCR was carried out by the following temperature cycle.

PCR Program

1. Step1	42 ^{O}C 1hr	cDNA synthesis
	35cycles of	
2. Step 2	94 ^{O}C – 1min	Denature
3. Step 3	55 ^{O}C – 1 min	Anneal
4. Step 4	72 ^{O}C – 1 min	Extension
5. Step 5	72^{O}C – 10 min	Final extension

DETECTION AND DOCUMENTATION OF RESULTS:

After the PCR is over, the products were visualized in Agarose gel electrophoresis stained with Ethidium bromide.

1.2% Agarose gel was prepared in 0.5 x TBE buffer, to this 2µl of Ethidium bromide was added and allowed to polymerize.

After polymerization, the gel was immersed in the gel tank with TBE buffer. 6 µl of 100bp DNA Ladder (GENEI) was used. 10µl of the PCR product (samples) was mixed with 2µl of loading dye.

The gel was electrophoresised at 70-80v (5v/sq.cm) till the dye reached the half of the gel tray.

The gel was removed and was placed on UV transilluminator.

Methodology VI

Single tube multiplex PCR analyis employing dengue sero type specific amplimers.

Rapid assay by which a second PCR, with specific primers allowing sero type identification (DNA Products) of different sizes according to the dengue sero type obtained.

RESULTS

A total of 125 suspected Dengue cases admitted in the pediatric and Medical Wards of Government Rajaji Hospital during November 2005 – June 2006 were analysed for the distribution of cases month wise and it was found that there were 10 cases in November (8%), 15 cases in December (12%), 23 cases in January (18.4%), 16 cases in February (12.8%), 22 cases in March (17.6%), 30 cases in April (24%), 5 cases in May (4%) and 4 cases in June (3.2%). Thus the distribution of cases from January – April found more than the other months. This is given in Table No 1.

TABLE 1

MONTH WISE PREVALENCE OF DENGUE

Sl. No.	Month	Number
1	November	10 (8%)
2	December	15 (12%)
3	January	23 (18.4%)
4	February	16 (12.8%)
5	March	22 (17.6%)
6	April	30 (24%)
7	May	5 (4%)
8	June	4 (3.21%)

The samples were analysed according to the place of collection especially from endemic and non endemic areas for dengue, and it was found that 30 samples (24%) were collected from endemic areas and 95 samples (76%) were collected from non endemic areas. This is given in Table 2.

TABLE 2

PREVALENCE OF DENGUE

Sl. No.	Dengue Endemic areas	Total samples collected	% of samples collected	Dengue Non – Endemic areas	Total samples collected	% of samples collected
1	Chatrapatty	8	26.6%	Valanthur	26	27.36%
2	Pilliarpatty	12	40%	Senthurai	34	35.78%
3	Virudhunagar	6	20%	Poosaripatty	18	18.94%
4	Kalaiyarkoil	4	13.3%	Chettikulam	17	17.89%
TOTAL		30	24%		95	76%

Among the 125 cases studied 72 were males (57.6%) and 53 were females (42.4%), thus it was found that the incidence was high in the males. The samples were further analysed agewise and sexwise and it was found that among the 72 males, 38 cases were in the 0-15 years age group (30.4%), 14 were in the 16-30 years group (11.2%), 12 were in the 31-45 years group (9.6%), 5 were in 46-60 years group (4%) and 3 were in more than 60 years age group (2.4%). Similarly among 53 females, 24 were in the age group 0-15 (19.2%), 13 were in the age group of 16-30(10.4%), 9 were in the age group of 31-45(7.2%), 5 were in the age group of 46-60 (4%) years and 2 were in the age group above 60 years (1.6%). It was found that the age group commonly involved in both sexes were between 0-15 years. This is given in Table 3.

TABLE 3

AGE WISE AND SEX WISE PREVALENCE OF DENGUE

Sl. No.	Age Group	Male	Female	Total
1	**0-15**	**38 (30.4%)**	**24 (19.2%)**	**62 (49.6%)**
2	16-30	14 (11.2%)	13 (10.4%)	27 (21.6%)
3	31-45	12 (9.6%)	9 (7.2%)	21 (16.8%)
4	46 – 60	5 (4%)	5 (4%)	10 (8%)
5	> 60	3 (2.4%)	2 (1.6%)	5 (4%)
Total		72 (57.6%)	53 (42.4%)	125 (100%)

All the 125 samples were further analysed according to the clinical manifestation and it was found that 117 cases had Dengue Fever (93.6%), 8 had Dengue Haemorrhagic Fever (6.4%), and there were no case with Dengue Shock Syndrome. **Thus more cases were found with clinical manifestation of dengue fever.** This is given in Table 4.

TABLE 4

CLINICAL MANIFESTATION WISE ANALYSIS OF DENGUE CASES

Sl. No.	CLINICAL MANIFESTATION	TOTAL NUMBER
1	**DENGUE FEVER**	**117 (93.6%)**
2	DENGUE HAEMORRHAGIC FEVER	8 (6.4%)
3	DENGUE SHOCK SYNDROME	-
TOTAL		125 (100%)

The samples were further analysed according to the symptoms and it was found that fever was present in almost all cases. Fever with headache in 101 (81.5%) cases, Fever with myalgia in 106 (84.5%), Fever with rash in 15 (12%) cases and Fever with

Haemorrhagic manifestation in 8 (6%) cases. **Thus it was found that fever with myalgia was the commonest symptom found in the cases.** This is given in Table 5.

TABLE 5

SYMPTOM WISE ANALYSIS OF DENGUE CASES

Sl. No.	SYMPTOMS	TOTAL
1	Fever with headache	101 (81%)
2	**Fever with myalgia**	**106 (84.5%)**
3	Fever with arthalgia	40 (32%)
4	Fever with Haemmorhagic manifestation	8 (6%)

The samples were further analysed according to the Clinical Signs and it was found that 9 (7%) had Splenomegaly, 10 (8%) had Hepatomegalyy, 25 (20%) had rash and 4 (3.7%) had a positive Tourniquet test, and 61.4% cases showed no signs. **Thus rash was seen as the commonest clinical sign.** This is given in Table No. 6

TABLE 6

SIGNS ELICITED

Sl. No.	SIGNS	TOTAL
1	Splenomegaly	9 (7%)
2	Hepatomegaly	10 (8%)
3	**Rash**	**25 (20%)**
4	Tourniquet test	4 (3.6%)
5	No Signs	77 (61.4%)

The analysis of samples for the presence of IgM/IgG antibodies by BIAS-3 Dengue IgG/IgM Immuno Assay System showed that out of 125

samples 15 (12%) were positive for IgM antibodies and 4 (3.2%) were positive for IgG antibodies. This is given in Table No. 7.

TABLE 7

DETECTION OF IgM / IgG Antibodies by BIAS-3 Kit

ANTIBODIES	POSITIVE
IgM	15 (12%)
IgG	4 (3.2%)

Further analysis of the 15 IgM positive cases showed that 14 belonged to the dengue fever category and 1 patient belonged to Dengue haemorrhagic fever category, and the 4 IgG positive cases showed that all of them belonged to the Dengue fever group. This is shown in Table No. 8.

TABLE 8

CLINICAL STATE WISE ANALYSIS OF IgM / IgG ANTIBODIES BY BIAS-3 KIT

Sl.No.	CLINICAL STATE	IgM	IgG	TOTAL
1	**DF**	**14 (11.2%)**	**4 (3.2%)**	**18 (14.4%)**
2	**DHF**	**1 (0.8%)**	**0**	**1 (0.8%)**
3	**DSS**	**-**	**-**	**-**
TOTAL		**15 (12%)**	**4 (3.2%)**	**19 (15.2%)**

Sex wise and age wise analysis of IgM and IgG antibodies was done. Among the 15 IgM positive cases, 9 cases (60%) were males and 6 cases (40%) were females. Age wise analysis of the 15 IgM positive cases, showed that 10 cases were in the 0 – 15 years age group (66.6%) of which, 7 cases were males (46.6%) and 3 cases were females (20%).

Thus IgM antibodies were more commonly found in males in the age group 0 – 15 years. This is given in Table No. 9.

TABLE 9

AGE WISE AND SEX WISE ANALYSIS OF IgM POSITIVE

Sl. No.	Age Group	Male	Female	TOTAL
1	**0 – 15 years**	**7 (46.6%)**	**3 (20%)**	**10 (66.6%)**
2	16 – 30 years	2 (13.3%)	1 (6.66%)	3 (20%)
3	31 – 45 years	-	1 (6.66%)	1 (6.66%)
4	46 – 60 years	-	1 (6.66%)	1 (6.66%)
5	> 60 years	-	-	
TOTAL		9 (60%)	6 (40%)	15 (100%)

Similarly the IgG positive samples were analysed age wise and sex wise and it was found that among the 4 positive cases, 2 were males (50%) and 2 were females (50%). Among these 4 cases, 2 cases were found in the 16 – 30 years group (50%), 1 was male (25%) and 1 was female (25%). Similarly the remaining, 2 (50%) cases were found in the 31 – 45 years age group among these 1 was male (25%) and 1 was female (25%). **Thus IgG antibodies were found equally in males and females in the age group of 16 – 45 years.** This is given in table 10.

TABLE 10

AGE WISE AND SEX WISE ANALYSIS OF IgG POSITIVE

Sl. No.	Age Group	Male	Female	TOTAL
1	0 – 15 years	-	-	-
2	**16 – 30 years**	**1 (25%)**	**1 (25%)**	**2 (50%)**
3	**31 – 45 years**	**1 (25%)**	**1 (25%)**	**2 (50%)**
4	46 – 60 years	-	-	-
5	> 60 years	-	-	-
TOTAL		2 (50%)	2 (50%)	4 (100%)

The 19 positive samples by BIAS – 3 system were further analysed for the confirmation of IgM positivity by Pan Bio Dengue IgM capture ELISA and it was found that all the 15 IgM positive by BIAS – 3 dengue IgG / IgM Immuno assay were positive by Pan Bio IgM capture ELISA, and the 4 IgG positive were negative by PAN BIO IgM capture ELISA. This is given in Table 11.

TABLE 11

RESULTS OF IgM BIAS – 3 KIT AND PAN BIO IgM CAPTURE ELISA

Sl. No.	Kit used	Total No. of Tested	Number of Positive		
			IgM	IgG	Total
1	BIAS – 3 Dengue IgG / IgM Immunoassay	125	15 (12%)	4 (3.2%)	19 (15.2%)
2	Pan Bio IgM Dengue Capture ELISA	19	15 (78.9%)	-	15 (78.9%)

The 15 IgM positive sera samples were injected intra cerebrally into suckling mice and it was found that 10 out of 15 positive samples produced hunch back, less intake of food and fine tremors leading to paralysis within 4 – 5 days of post inoculation. 2 samples produced only weakness but no paralysis or convulsions and 3 samples produced only prostration but no paralysis. This is given Table No. 12

TABLE 12
RESULTS OF VIRUS ISOLATION

SL. NO.	TOTAL	TYPICAL (SICK + PARALYSIS + CONVULSIONS)	WEAKNESS + NO PARALYSIS + NO CONVULSIONS	ONLY PROSTRATION NO CONVULSIONS
1	15	10 (66.6%)	2 (13.3%)	3 (20%)

The HA titre of the brain tissue was determined in the 10 samples which showed positive symptoms and the titres were found as follows.

TABLE 13

HAEM AGGULITINATION TITRES

Sample No.	Titres					
	10	10-40	40-80	80-160	160-320	> 320
1	-	-	-	Present	-	-
2	-	-	-	-	Present	-
3	-	-	-	Present	-	-
4	-	-	-	Present	-	-
5	-	-	-	Present	-	-
6	-	-	-	-	-	Present
7	-	-	-	Present	-	-
8	-	-	-	-	Present	-
9	-	-	-	-	-	Present
10	-	-	-	Present	-	-
TOTAL	0	0	0	6 (60%)	2 (20%)	2 (20%)

Among the 10 samples, 6 showed titres between 80 – 160 (60%), 2 showed titres between 160 – 320 (20%) and 2 showed titres more than 320 (20%). Thus 4 samples showed titres ranging from 160 – 320. All the 10 samples were further processed by RT PCR to find the base pair region of the Dengue virus. It was found that out of 10 samples, 4 samples which showed titres more than 160, showed amplification at 511 bp C-Pre M region denoting that it is a dengue complex. These dengue complex positive samples were analysed age wise, sex wise and according to clinical state and it was found that all the four samples were from males in the age group ranging from 3 – 9 years, 3 of them showed

fever and one showed haemorrhagic manifestations. This is given in Table No. 14.

TABLE 14

CLINICAL STATE AND PCR ANALYSIS

SL. NO.	CLINICAL STATE	AGE/ SEX	RT-PCR 511 bp	% POSITIVE
1	DF	37 / F	Negative	40%
2	DF	7 / M	Positive	
3	DF	5 / F	Negative	
4	DF	45 / F	Negative	
5	DF	16 / M	Negative	
6	DHF	9 / M	Positive	
7	DF	41 / M	Negative	
8	DF	8 / M	Positive	
9	DF	3 / M	Positive	
10	DF	30 / F	Negative	

These Dengue complex positive samples were further analysed by using single tube multiplex PCR to find out the serotype of the complex using Dengue serotype specific amplimers. All the four samples analysed showed the presence of Dengue 3 specific 290 bp amplicon denoting the serotype belonged to Type 3. This is given in Table No. 15.

TABLE 15

SERO TYPE ANALYSIS

SL. NO.	CLINICAL STATE	AGE/ SEX	RT-PCR 511 bp	SERO TYPE (290 bp amplicon)
1	DF	7 / M	Positive	TYPE III
2	DF	8 / M	Positive	TYPE III
3	DHF	9 / M	Positive	TYPE III
4	DF	3 / M	Positive	TYPE III

DISCUSSION

The study of Prevalence of dengue sero type in Madurai by molecular methods involved 125 suspected dengue cases admitted in Government Rajaji Hospital during November 2005 – June 2006.

In the present study, it was found that 57.6% of the affected population were males and 42.4% were females. **Gadia R R et al** [21] in his study had shown that 70% of the affected population were males. Similar studies of **Gupta et al** [30] also demonstrated the incidence of dengue infection were more in males, the ratio being 2.1:1. These findings support the present study, whereas the study by **Rita Maria et al** [59] showed that the incidence was more in females than males, the ratio being 1:1.08. The study by **Philip Samuel et al** [55] revealed no sex difference in these cases. **Susan Shepherd et al** [71] says that the dengue infection occur irrespective of sex. But in the present study there were more number of males than females, this might be due to the selection of more number of cases from villages, where the dressing culture of the males favoured the biting by the mosquitoes while compared to the females.

In the present study agewise analysis of the samples revealed that the **commonest age group affected was 0 – 15 years in both males and females.** Similar study by **Victor J et al and Soundaravally et al** [73, 67]

also revealed the occurrence of dengue infection commonly in the age group 0 – 15 years, the percentage being 65% and 34.2% respectively. This might be due to the endemicity of the infection in many of our population, where the older and many younger inhabitants are immune, where as the children between 0 – 15 years are more affected.

In the present study it was found that the number of samples collected from January to April were more, with the **maximum number of cases in April 2006 (24%)**. This period is neither a pre monsoon nor a post monsoon. **Thakara et al** [72] in his study stated that increased number of cases in dengue infection were mainly related to the cyclic changes in weather which favoured the breeding of A. aegypti. **G S Chouhan et al** [13] of Rajasthan also demonstrated more number of sample collection during the pre monsoon period. A study by **Emran Pin Yunus et al** [20] of Bangladesh, **Mahbubur Rahman et al** [50] also revealed the incidence was more common in the post monsoon season for obvious reasons. Even though these studies do not correlate with my studies, it was found that we had two unusual rains during December 2005 and March 2006, perhaps these might be the reason for the increased number of cases in January and April, which were the post raining months, in which the breeding of A. aegypti mosquitoes would have been encouraged.

In the present study it was found that **dengue fever** with out clinical manifestation of haemorrhage were common (93.6%) followed by dengue haemorrhagic fever (6.4%) and there was no case of dengue shock syndrome. The study by **Manoj Kumar et al [51],** New Delhi showed an incidence of dengue fever in 75% cases, dengue haemorrhagic fever in 21.9% cases and dengue shock syndrome in 3.1% of cases. The study by **Mahbubur Rahman et al [50]** also revealed 60% dengue fever followed by 39.2% dengue haemorrhagic fever and 0.6% dengue shock syndrome. All these studies favour our findings except that there was no dengue shock syndrome reported in my study. As most of my cases were from the non endemic areas of dengue infection, the antibody dependent enhancement and replication of dengue virus in cells of mono nuclear cell lineage would not have occurred, hence no secretion of vasoactive mediators, resulting in shock.

The clinical symptom of dengue commonly involved in my study was **fever with myalgia 84.5%,** whereas fever with headache accounted for 81% and fever with arthralgia accounted for 32% and fever with haemorrhagic manifestation accounted for 6%. The study by **Gadia R R et al [21]** also revealed that most of the cases in their study showed symptoms of fever with headache 87%, fever with myalgia 70%, fever with arthralgia 54.9%. This is in support of my findings denoting **fever with myalgia, is the most common symptom in dengue cases.**

In the present study, the commonest sign elicited in the dengue cases were rash 20% followed by splenomegaly in 7%, hepatomegaly in 8%, positive tourniquet test in 3.2%. Similarly the study by **Kabilan L et al** [44] demonstrated that 55.2% of his cases had rashes. The study by **Gadia R R et al** [21] also revealed the presence of rash in 21% of cases, splenomegaly in 11% and hepatomegaly in 17% and the positive tourniquet test in 11%. All these studies are in support of our findings, denoting the **commonest signs elicited in dengue infection was the rash.**

In the present study IgM antibodies were demonstrated in 15 (12%) and IgG was demonstrated in 4 (3.2%). 14 out of the 15 IgM positives belonged to the dengue fever category (11.2%) and 1 belonged to the dengue haemorrhagic fever category (0.8%) and all the IgG positive cases belonged to the dengue fever group 3.2%. **Thakara J P et al** [72] in his studies demonstrated that 14.6% cases were positive for IgM antibodies. Similarly, study by **Philip Samuel et al** [55] also revealed 14.4% positive for IgM antibodies. All these IgM positivity were detected in dengue fever cases. The study by **Sathish et al** [62]**,** CMC also showed 22.7% positivity. All these studies are in support of our study, revealing that **IgM antibody** is found more commonly in dengue fever cases. In the present study it was found that IgM antibodies were commonly found in

males in the age group 0-15 years (46.6%), whereas the IgG antibody was found equally in males and females in the age group of 16 – 35 years.

The appearance of IgG antibodies in the older age group may be due to the secondary immune response elicited by these cases, due to prior infection, where as the younger age group, in whom the primary immune response is common, IgM antibodies were elicited. This is explained by **Ravulapalli Anandha Rao et al** [58].

In the present study IgM capture ELISA has proved to be the best confirmatory test by eliciting 78% positivity in the IgM positive cases screened by BIAS 3 kit. Similarly study by **Soundaravally et al** [67] showed 85.2% positivity by Mac ELISA. But **Rita Maria et al** [59] showed IgM positive by Mac ELISA to be 53.3% and study by **De Paulo et al** [16] showed a positivity of 27.9%. The variation in the positive rate is due to the usage of IgM capture ELISA either as a screening or confirmatory methodology. In our study we used it as a **confirmatory methodology**, whereas the other studies used it as a screening methodology.

In the present study it was shown, that virus was isolated in 10 out of 15 IgM positive samples by Intracerebral inoculation into suckling mice (66.6%). Similar studies by **Parida M M et al** [54] demonstrated 80% virus isolation in suckling mice. This is in support of our finding, whereas **Ratho R K et al** [57]**,** Chandigarh showed the isolation rate from suckling mice was 23.8%. This variation in isolation rate might be due to

the selection of animals showing positive symptoms. In my study I have selected only the sick animals with paralysis and convulsion. Although some animals showed weakness and prostration as they did not produce paralysis or convulsions, these animals were not considered.

In the present study, haemagglutination (**HA**) was used as the confirmatory test for the identification of the dengue virus in suckling mice brain and HA titres ranging from 80 to 320 were taken as positive. All the brain tissues inoculated with IgM positive samples showed these titres. The confirmatory test for the dengue antigen detection varied in various studies. **Ratho R K et al** [57] has used indirect immuno fluorescence test as a confirmatory test. **Philip Samuel et al** [55] used mosquito inoculation technique followed by direct immuno fluorescence to be the confirmatory test for dengue antigen.

In the present study, out of the 10 IgM positive samples subjected to RT-PCR, only 4 yielded amplification of 511bp C-Pre M region of dengue virus (40%). Out of the 4 RT-PCR positive samples, 3 of them presented with dengue fever and 1 showed haemorrhagic manifestation. Similar study by **Manoj Kumar et al** [51] revealed 21.8% positive by RT-PCR and study by **Rita Mario et al** [59] revealed 32% positive by RT-PCR and the study by **Ratho R R et al** [57] revealed 78.9% positivity by RT-PCR. As our samples were collected during 5 – 10 days of onset of symptoms, detectable level of dengue specific IgM antibodies, appearing

after 4 – 5 days was demonstrated in most of our samples, whereas the molecular test showed less number of positivity because the ideal time for collection of samples for PCR testing was between 1 – 5 days as described by **Manoj Kumar et al [51].**

In the present study it was found that there was an emergence of a different sub type of dengue virus at Madurai. In the present study dengue – 3 was seen as the predominant type whereas in our previous studies we have demonstrated dengue type 2 as the predominant type. **Manoj Kumar et al [51]** had already explained the replacement of one type of dengue by the other by the fact that in 1971, dengue 2 was introduced in the Pacific followed by dengue 1 in 1975 and dengue 4 in 1979 and dengue 3 in 1980. Dengue 3 had been already reported from various parts of India, South East Asian Countries and in the Americas. The first reported evidence of dengue 3 in Delhi was in 1970, in Jamaica was in 1963, in Columbia was in 1970, in Pacific Island was in 1980 and Americas was in 1994.

The study of **Dash P K et al [15],** Defence Research and Development Establishment, Gwalior, 2003 showed the implication of Dengue type 3, in an out break of dengue infection that occurred in northern India during October – December 2003. The study by **William B Messer et al [78]** showed that the dengue type 3 was responsible for the out breaks in Sri Lanka and Bangladesh (2003). This confirms the

reemergence of dengue type 3 in a dominant form in the Indian subcontinent. **Soundravally R et al** [67] also showed the presence of type 3 in the dengue out break that occurred in October 2003 – February 2004. Sequencing of the amplicon from the isolated dengue type 3 confirmed the sero type and phylogenetic analysis based on nucleotide sequence showed the closeness of the Pondicherry strains to Peru and Taiwan strains.

Ramamurty N et al [56] in their study carried out in Chennai showed the presence of both sero types 2 and 3. **Rita Maria Riberio Nouguiria et al** [59] from Brazil has also reported dengue virus type 3 in the summer epidemic of Rio de Janeiro. All these study reports are similar to our findings.

SUMMARY

The study of prevalence of dengue sero type in Madurai by molecular methods was aimed at finding the sero type of dengue prevalent in Madurai during the period November 2005 – June 2006, by molecular methodology. Out of 125 dengue suspected dengue cases admitted at Government Rajaji Hospital, Madurai, it was found that more number of cases were admitted from January – April 2006, which was neither a pre monsoon nor post monsoon period, but due to the unusual rains in December 2005 and March 2006, leading to more number of cases reported during this period. In the study population 57.6% were males and 30.4% of these males were in the age group of 0 – 15 years.

The commonest symptom present in 84.5% of the study population was fever with myalgia and 93.6% cases come under the category of dengue fever and 20% showed the sign of rashes.

The IgM antibody study by both spot ELISA and IgM capture ELISA revealed that 12% were positive for IgM antibodies and 11.2% of these positives presented with dengue fever and 0.8% presented with Dengue haemorrhagic fever.

The IgM positive samples, inoculated in suckling mice revealed that 10 out of 15 (66%) brain tissues demonstrated dengue virus.

The confirmation of dengue virus, in the brain materials was done by HA technique showing titres ranging from 80 to 320. By RT- PCR, 4 out of 10 IgM positive inoculated brain samples (40%) showed the amplifications of 511bp C-Pre M- region of dengue virus. By single tube multiplex PCR analysis employing dengue sero type specific amplimers, all these 4 samples revealed 290 bp amplicons, denoting that this was dengue sero type 3.

CONCLUSION

The study of prevalence of dengue sero type in Madurai by molecular methods revealed the following:

- Dengue cases were common during January 2006 – April 2006, due to the unusual rains in December 2005 and March 2006.
- More number of males in the age group 0 – 15 years were affected.
- The commonest symptom demonstrated was fever with myalgia and commonest sign was rashes.
- The spot IgG / IgM and IgM capture ELISA revealed that 12% of the affected were positive for IgM antibodies.
- Dengue virus antigen was prepared from the brain of the suckling mice by inoculating the IgM positive samples and the antigen titre was confirmed by HA technique taking into account 80 – 320 as the range of the titre value.
- These isolated viruses were subjected to RT-PCR and 40% of them showed amplification of 511 C-Pre M- region confirming the presence of dengue virus.
- The sero type of dengue virus was found out by single tube multiplex PCR analysis employing dengue sero type specific amplicons demonstrating 290 bp amplicons suggestive of dengue sero type 3.

- In the present study dengue 3 seems to be a emerging infection in Madurai population, because since 1986 the arboviral laboratory of Madurai Medical College demonstrated only dengue 2 in Madurai population. The common symptom observed was dengue fever, now another sero type is emerging and there is a possibility of dengue haemorrhagic fever and dengue shock syndrome. So the clinicians must be aware of this fact, if any haemorrhagic fever occurs they should keep the fact in mind of dengue in treating these cases.

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PROFORMA

Case Investigation Form

Date:

Patient's Name: Patient's I.D. No.:

Age / Sex:

Father's / Husband's Name:

Address: Epidemic / Non-Epidemic

Date of on set of illness:

Date of hospitalization:

Occupation:

Clinical signs & symptoms (with duration)

Treatment History:

Results of previous investigations (if any):

Any other relevant information:

Specimen details:

Nature of specimen (s)	Date of collection	Investigation required

Details of sender:

Signature: Address of sender:

Name of sender: E-mail:

Fax:

PROFORMA
Laboratory Form

Date:

Patient's Name: Patient's I.D. No.:

Age / Sex:

Laboratory Reference No.:

Specimen details:

Type of Specimen	Date of Collection	Date of Receipt in lab	Type of test	Remarks (if any)	Result

Interpretation:

Details of Investigator:

Name:

Signature

Telephone No.:

Address:

ANNEXURE - I

PREPARATION OF REAGENTS

I. Borate Saline pH 9.0

1.5M NaCl	-	160 ml
0.5M H_3BO_3	-	200 ml
1N NaOH	-	47 ml

Add double D.W. to make 2 litre, pH 9.0 (if pH is not 9.0 then add 0.5M H_3BO_3or IN NaOH to adjust)

II. 0.4% percent Bovine Albumin in Borate Saline (BABS) pH 9.0

Bovine Albumin	-	4.0 gm
Borate Saline pH 9	-	100 ml

Keep overnight in cold and adjust pH to 9.0 with 1M NaOH. Take 100 ml of this solution and add to 900 ml of borate saline solution, pH 9.0.

III. 25% Percent Kaolin in Borate Saline pH 9.0

Kaolin (acid washed)	-	25 gm
Borate Saline pH 9.0	-	100 ml

Stir Borate Saline on magnetic stirrer and add Kaolin powder slowly till uniform suspension is obtained.

IV. 0.9% % NaCl

NaCl	-	9 gm
Double distilled water (DDW)	-	1000 ml

Autoclave at 15 Ibs for 15 minutes.

V. Acid Citrate Dextrose (ACD)

Sodium Citrate	-	11.26 gm
Citric Acid	-	4.0 gm
Dextrose	-	11.0 gm
DDW	-	500 ml

Autoclave at 15 Ibs for 10 minutes.

VI. Dextrose Gelatin Veronal

A. Veronal (Diethyl barbituric acid)	-	0.580 gm
Gelatin	-	0.600 gm
Hot D.D.W.	-	250 ml
B. Sodium Barbitone	-	0.380 gm
$MgSO_4 7H_2O$	-	0.120 gm
$CaCl_2$ (anhydrous)	-	0.020 gm
NaCl	-	8.5 gm
Dextrose	-	10.0 gm
Merthiolate	-	0.1 gm
D.D.W	-	750 ml

Mix A and B solutions. Autoclave at 10 Ibs for 10 minutes.

VIII. Preparation of Reagents

Collection and Washing of goose RBC

Collection of RBC

Goose RBC are collected under sterile condition. Take 125 ml of DGV in a 250ml flask.

1. Take 10 ml ACD in a sterile tube.
2. Withdraw 7.5 ml ACD in to 50ml syringe fitted with a 20 gauze needle.
3. Collect 50ml goose blood from the wing or jugular vein.
4. Remove needle from syringe and transfer blood from syringe to flask with DGV.
5. Mix well.
6. Store at $4^{O}C$ (can be kept for up to 2 weeks).

Washing of RBC

1. Centrifuge required quantity of RBC suspension in a graduated centrifuge tube for six minutes at 2000 RPM.
2. Aspirate off the supernatant fluid and buffy layer of WBC with Pasteur pipette attached to a rubber bulb without disturbing the packed RBC.
3. Add 2.0 ml of normal saline (N.S)
4. Mix gently with Pasteur pipette to resuspend cells and additional quantity of N.S. to bring it to be original volume.
5. Centrifuge at room temperature for 3 minutes at 2000 RPM.
6. Repeat step 2,3,4 and 5.
7. Centrifuge at 2000 RPM for 6 minutes.
8. Read volume of packed RBC.
9. Remove supernatant fluid.
10. Add NaCl to make a 10% suspension of RBC and mix. The cells are ready for use in HI. (Use washed and packed cells for adsorption of sera).

ETHICAL CLEARANCE CERTIFICATE

I **DR. M. SHANTHI., M.D., (Pharmacology),** Dean & Chairman Animal Ethical Committee, Madurai Medical College, Madurai, hereby endorse ethical clearance to the proposal.

" GENE SEQUENCE BASED MOLECULAR CHARACTERISATION OF DENGUE VIRUS IN SUSPECTED CASES IN MADURAI "

Submitted by

Dr. S. LALLITHA
Post Graduate in Microbiology,
Institute of Microbiology,
Madurai Medical College,
Madurai.

The study design did not violate the regulations and guidelines prescribed by ICMR and are within the permitted norms of animal experimentation in this country. The outcome of the study may be beneficial in understanding the sero type of Dengue Virus prevalent in Madurai.

Date:
Place:

மதுரை மருத்துவக் கல்லூரி
19 APR 2006
மதுரை - 625 020

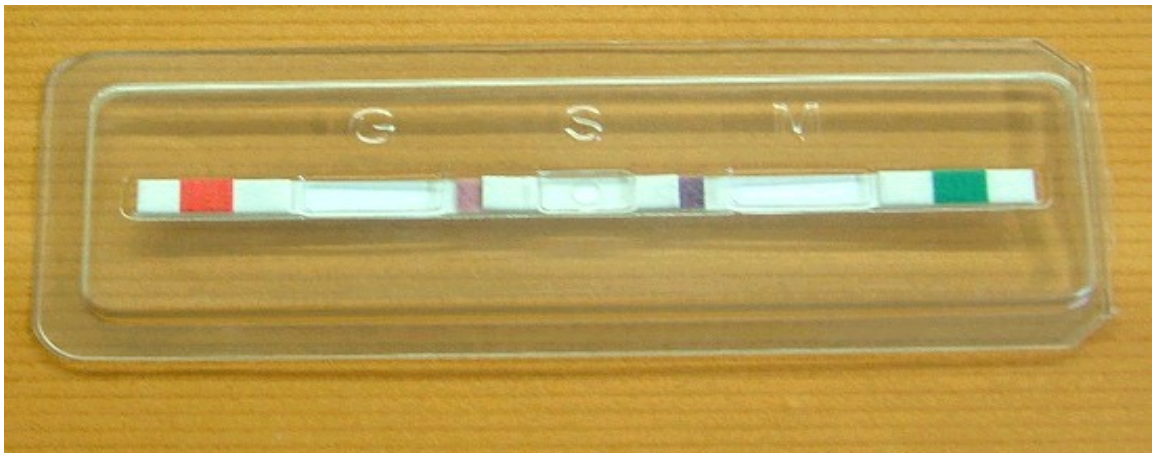
Office Seal

M. Shanthi
19.4.06

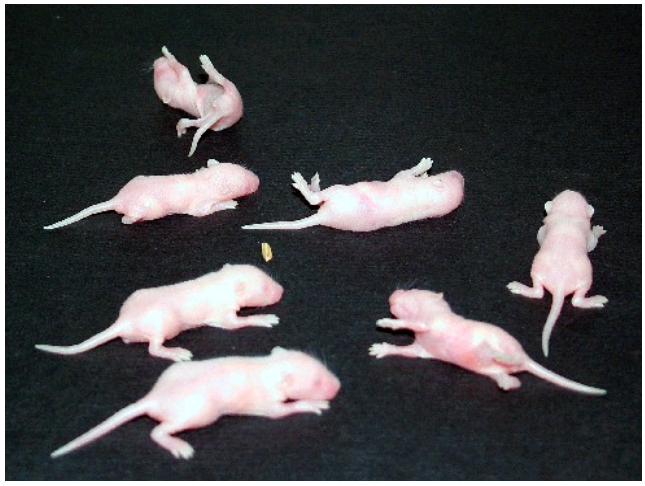
DEAN & CHAIRMAN

BIAS 3 DENGUE IgM / IgG KIT

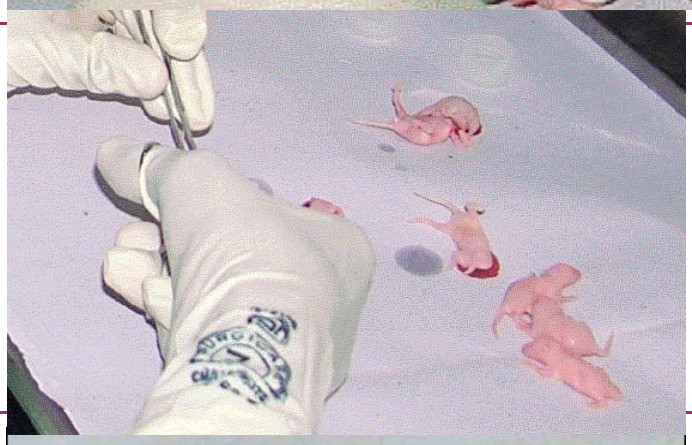
BIAS 3 DENGUE IgM KIT



SUCKLING MICE SHOWING SYMPTOMS



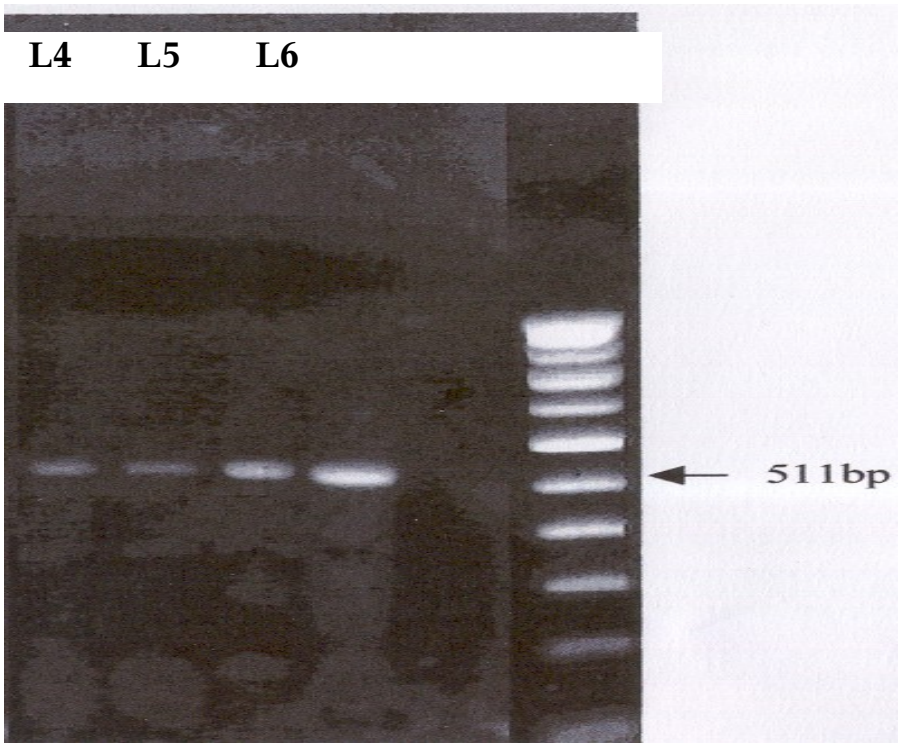
HARVESTING OF BRAIN TISSUES



THERMO CYCLER

GEL DOCUMENTATION SYSTEM

PCR POSITIVE SAMPLES SHOWING BANDS AT 511 bp



L1 : Positive Sample

L2 : Positive Sample

L3 : Positive Sample

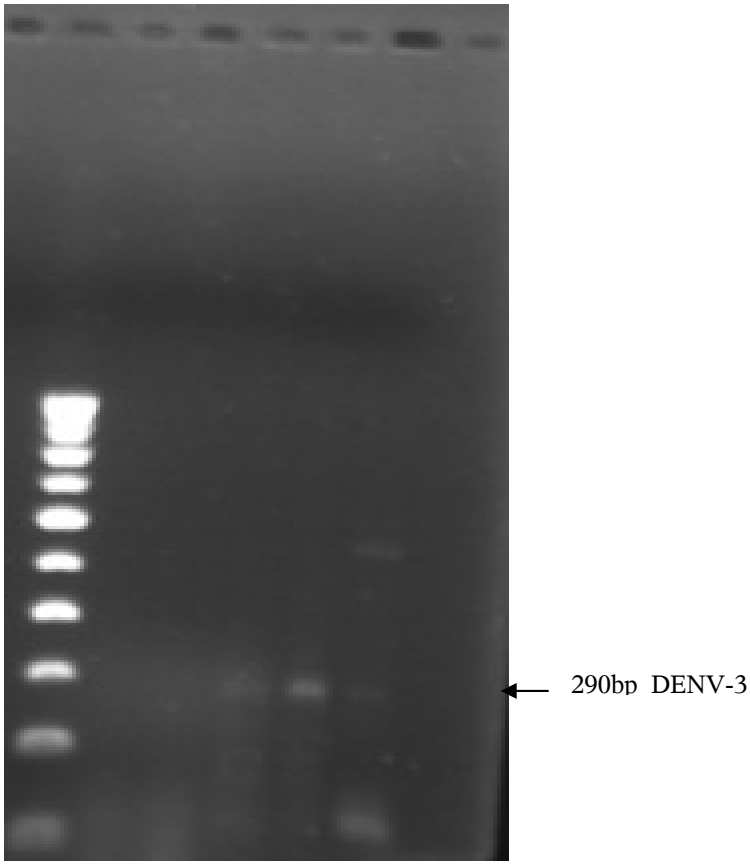
L4 : Positive Sample

L5 : Negative Control

L6 : Ladder [100 bp]

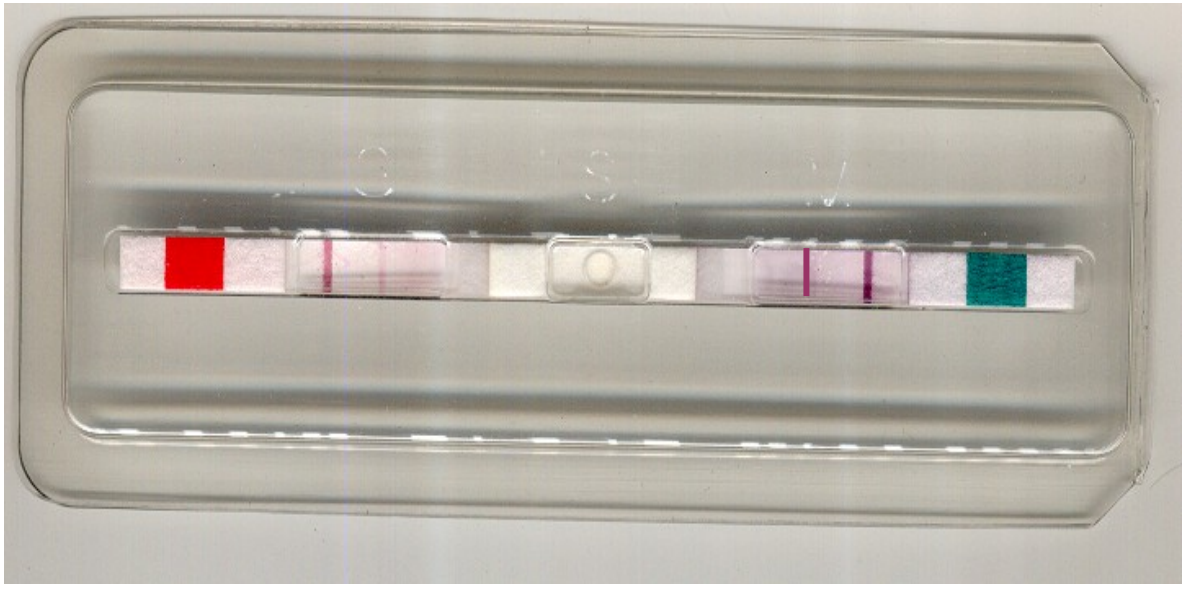
SINGLE TUBE MULTIPLEX PCR ANALYSIS FOR SERO TYPING OF DENGUE VIRUS

L1 L2 L3 L4 L5 L6 L7

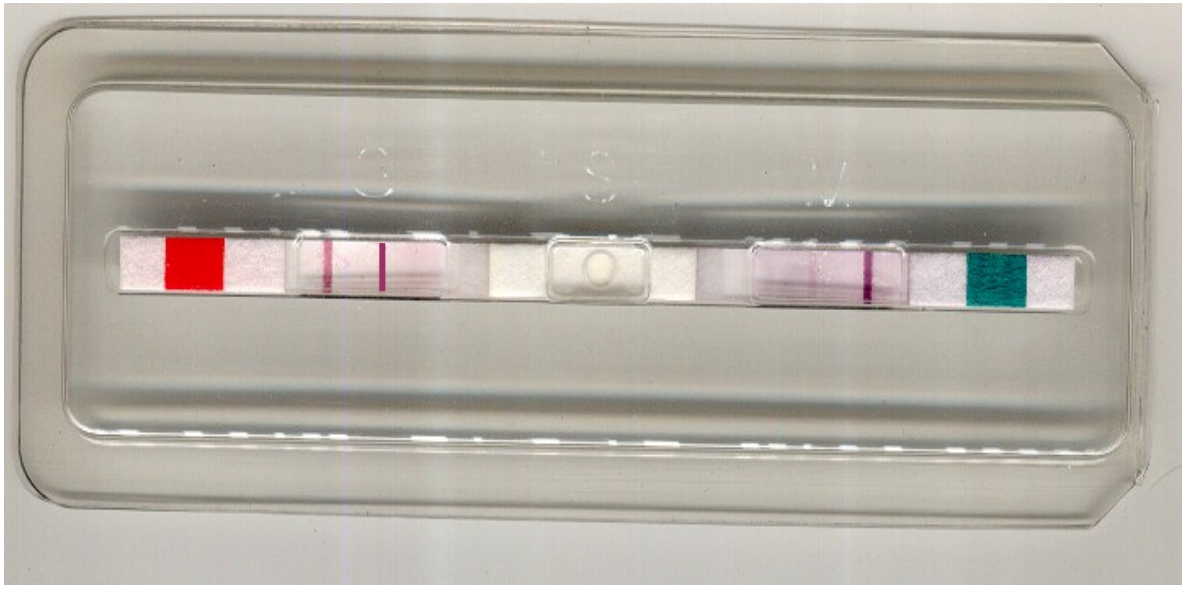


L1- MARKER
L2- NEGATIVE CONTROL
L3- DENV-3
L4- DENV-3
L5- DENV-3
L6- DENV-3

BIAS 3 IgM / IgG KIT SHOWING IgM POSITIVE



BIAS 3 IgM / IgG KIT SHOWING IgG POSITIVE



RESULTS OF HAEMAGGLUTINATION TEST